LETTER TO JMG

Gender specific association of aldosterone synthase gene polymorphism with renal survival in patients with IgA nephropathy

J Song, I Narita, S Goto, N Saito, K Omori, F Sato, J Ajiro, D Saga, D Kondo, M Sakatsume, F Gejyo

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Immunoglobulin A nephropathy (IgAN), which is the most prevalent form of primary glomerulonephritis and one of the major causes of end stage renal disease (ESRD), has a variable clinical course.^{1–3} Poor prognostic factors for the progression of renal dysfunction in IgAN have been identified as high blood pressure, heavy proteinuria, and a severe histopathological appearance of the renal biopsy.^{4–5} In addition to these prognostic factors, it has been proposed that several genetic backgrounds are associated with a susceptibility to ESRD in patients with IgAN.^{6–7}

The renin-angiotensin-aldosterone system is an important regulator of blood pressure and plays a central role in the development and progression of end organ damage. Polymorphisms in genes that encode components of this system have been reported to be associated with physiological risk factors for progressive renal dysfunction in IgAN. The most consistent of these is the angiotensinogen (*AGT*) gene, which is associated with essential hypertension and with an increased risk of cardiovascular diseases and renal failure.^{8–10} A deletion polymorphism in the angiotensin converting enzyme (*ACE*) gene influences the circulating ACE levels, and although it has little effect on blood pressure, it has been associated with an increased risk of cardiovascular diseases in some but not all studies.^{11–13}

Aldosterone is one of the main effectors of the renin-angiotensin system¹⁴ and has classically been thought to act as a regulator for the absorption of Na and water, as well as the excretion of K in normal physiology, and as a mediator of oedema in numerous disease states. However, it is now well recognised that the actions of aldosterone are not limited to effects on ion transport in epithelial tissue, and its important role in cardiovascular disease involves non-epithelial tissues.^{15–16} Recently, it has been shown that aldosterone has a number of deleterious effects on the cardiovascular system, including necrosis and fibrosis, vascular stiffening and injury, reduced fibrinolysis, endothelial dysfunction, and catecholamine release.

Aldosterone secretion is regulated largely by the expression level of the final enzyme required for its biosynthesis, aldosterone synthase (*CYP11B2*).¹⁷ Expression of *CYP11B2* is regulated by angiotensin II through cAMP dependent modulation of the gene promoter region, which contains a variety of control factors.^{18–19} Therefore, genetic variants in *CYP11B2*, which may be associated with the biosynthesis of aldosterone in local tissue, may also affect the progression of renal dysfunction in primary glomerulonephritis.

One potentially interesting variant of *CYP11B2* is located in the 5' flanking region of the gene, with a C or T at 344 nucleotides upstream from the start of translation (C-344T) within a binding site for the transcription factor steroidogenic factor-1 (SF-1).²⁰ This genetic polymorphism has been reported to be associated with the serum level of aldosterone, as well as the left ventricular size, its function, and myocardial infarction

Key points

- Aldosterone has a number of deleterious effects on the cardiovascular system, including necrosis and fibrosis, vascular stiffening and injury, reduced fibrinolysis, endothelial dysfunction, and catecholamine release. The C-344T polymorphism in the aldosterone synthase gene (*CYP11B2*), which is associated with circulating aldosterone concentration, has recently been proposed as a genetic candidate in cardiovascular disease such as hypertension. However, its role in the progression of renal dysfunction in IgA nephropathy (IgAN) remains to be investigated.
- We analysed the *CYP11B2* C-344T polymorphism in 271 patients with biopsy proven IgAN and investigated a possible association between the polymorphism and the prognosis of renal function using the Kaplan-Meier method and Cox proportional hazards regression model.
- At the time of renal biopsy there was no difference among the genotypes in any clinical manifestations, including age, gender, urinary protein excretion, and blood pressure. Predictive risk factors for progressive renal dysfunction were identified as hypertension, urinary protein of more than 1.0 g/day, and no administration of angiotensin converting enzyme inhibitor (ACEI) or angiotensin receptor blocker (ARB) in both female and male patients.
- Even after adjusting for these risk factors, the CC genotype of *CYP11B2* was a significant risk factor only in female patients with a hazard ratio (HR) of 4.284 ($p=0.0022$). In contrast, no effect at all was observed in male patients ($HR=0.788$, $p=0.6269$).
- Genetic variation in or near the *CYP11B2* gene is a possible genetic marker for the progression of renal dysfunction in females with IgAN but not males.

independently of blood pressure.^{21–23} In contrast, a previous study in patients with kidney disease, in which a variety of primary kidney diseases for ESRD were included in the analysis, did not show any significant effect of the *CYP11B2* polymorphism on the progression of renal dysfunction.⁹ Thus, the possible role of the *CYP11B2* polymorphism on renal survival in patients with IgAN remains to be analysed fully.

In this study, we investigated the possible role of the *CYP11B2* C-344T polymorphism in renal prognosis in Japanese patients with IgAN.

Table 1 Clinical characteristics of the patients and their comparison by each genotype in women and men

	Women (n=143)					Men (n=128)				
	Genotype of <i>CYP11B2</i>					Genotype of <i>CYP11B2</i>				
	Total n=271	TT n=64	CT n=63	CC n=16	P value	TT n=52	CT n=64	CC n=12	P value	
At the time of renal biopsy										
Age (year)	37.0 (13.4)	38.9 (13.4)	36.0 (13.0)	35.6 (14.0)	NS	38.4 (12.4)	34.4 (14.3)	41.0 (12.7)	NS	
Urinary protein excretion (g/day)	1.36 (1.35)	1.17 (1.20)	1.02 (1.17)	1.63 (1.39)	NS	1.62 (1.71)	1.56 (1.30)	1.53 (0.98)	NS	
Serum creatinine (mg/dl)	0.99 (0.62)	0.84 (0.32)	0.77 (0.26)	0.91 (0.52)	NS	1.24 (0.75)	1.16 (0.87)	1.14 (0.27)	NS	
Creatinine clearance (ml/min)	89.2 (33.1)	83.2 (25.6)	96.6 (33.5)	82.9 (33.4)	NS	88.2 (33.4)	92.0 (39.7)	82.5 (24.6)	NS	
Blood pressure (mm Hg)										
Systolic	127.6 (18.3)	126.6 (18.7)	123.6 (17.4)	130.8 (25.9)	NS	130.1 (16.0)	128.4 (18.1)	133.4 (19.5)	NS	
Diastolic	77.1 (13.5)	76.4 (12.1)	74.8 (12.2)	76.9 (15.4)	NS	77.9 (15.0)	77.9 (14.4)	84.5 (13.2)	NS	
Incidence of hypertension (%)	36.4	39.1	22.0	35.7	NS	41.2	37.5	66.7	NS	
During observation										
Observed period (month)	92.0 (66.8)	102.1 (61.9)	86.9 (58.3)	61.1 (40.1)	0.0366	91.7 (80.6)	92.7 (74.1)	105.7 (48.3)	NS	
Incidence of progressive renal disease (%)	31.0	29.0	25.8	50.0	NS	29.4	34.4	41.7	NS	
Corticosteroid (%)	28.3	27.1	23.2	33.3	NS	32.7	28.3	33.3	NS	
ACEI/ARB (%)	40.8	41.3	33.9	50.0	NS	54.9	33.3	41.7	NS	
Blood pressure (mm Hg)										
Systolic	128.3 (16.7)	128.1 (17.3)	125.6 (17.8)	133.1 (22.7)	NS	127.6 (12.6)	129.5 (16.4)	132.8 (17.0)	NS	
Diastolic	77.4 (11.9)	76.8 (10.5)	75.9 (12.2)	78.9 (13.1)	NS	76.4 (11.6)	78.3 (12.7)	84.6 (11.7)	NS	
ACEI/ARB, angiotensin-I converting enzyme inhibitor and/or angiotensin receptor blocker; NS, not statistically significant. Values are mean (SD).										

Table 2 Allele frequencies in hypertensive and normotensive subjects

	Allele	Hypertensives	Normo-tensives	p value	χ^2
Total	-344C	66	117	0.8723	0.026
	-344T	132	227		
Women	-344C	26	69	0.2891	1.124
	-344T	64	127		
Men	-344C	40	48	0.4436	0.587
	-344T	68	100		

METHODS

Study subjects

Written informed consent was obtained from all patients. The ethics committee of the institution approved the protocol for the genetic study. The study included 271 patients with biopsy proven IgAN. In all cases, the diagnosis of IgAN was based on the immunofluorescent microscopy of a biopsy specimen, which showed dominant or co-dominant deposition of IgA in the glomerular mesangium. Patients with Schönlein-Henoch purpura and secondary IgAN such as hepatic glomerulosclerosis or rheumatoid arthritis were excluded from the study. Clinical characteristics of the patients at the time of diagnosis including age, gender, urinary protein excretion (g/day), level of serum creatinine (sCr, mg/dl), and 24 hour creatinine clearance (ml/min) were investigated. Hypertension was defined by the use of one or more antihypertensive medications and/or a blood pressure greater than or equal to 140 mm Hg systolic or 90 mm Hg diastolic. The primary end point (PRD, progressive renal disease) was defined as the date when the sCr level was double that at the time of diagnosis, or when patients underwent their first renal replacement therapy. The mean duration of observation was 92.0 (SD 66.8) months. The administration of glucocorticoids, antihypertensive agents, angiotensin converting enzyme inhibitors (ACEI), and angiotensin II receptor blocker (ARB) was also recorded for each patient.

DNA preparation and genotype determination

Genomic DNA was extracted from the peripheral blood cells of patients by an automatic DNA isolation system (NA-1000, Kurabo, Osaka, Japan). The genotype of *CYP11B2* C-344T was determined by the PCR-RFLP method using restriction endonuclease *HaeIII* (Takara, Kyoto, Japan) as described previously.²¹ Primers used for the PCR reaction were 5'-CAG GAG GAG ACC CCA TGT GAC-3' (sense) and 5'-CCT CCA CCC TGT TCA GCC C-3' (antisense). The reaction mixture contained 1 × PCR buffer, 1.5 mmol/l MgCl₂, 200 mmol/l deoxynucleotide triphosphates (dNTPs), 1 unit *Taq* DNA polymerase (Takara, Kyoto, Japan), 10 pmol of each primer, and 50-100 ng genomic DNA. The PCR amplification reaction consisted of a cycle at 95°C for five minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing at 67°C for 15 seconds, and extension at 72°C for 30 seconds. A final extension was performed at 72°C for five minutes. The 537 bp PCR products were digested with restriction endonuclease *HaeIII* (Takara), and electrophoresed on a 2.5% agarose gel.

Statistical analysis

Statview 5.0 statistical software (Abacus Concepts Inc, Berkeley, CA, USA) was used for statistical analyses on a Macintosh G4 computer. Chi-square analysis was used when comparing allele frequencies and categorical variables between the groups. Continuous variables were compared using the Mann-Whitney U test or Kruskal-Wallis analysis of variance.

Figure 1 The renal survival rate in IgAN patients with each genotype of the *CYP11B2* C-344T polymorphism. The renal survival rate of patients with the CC genotype (solid line, n=28) was significantly worse than that in other genotypes (dotted line, CT, n=127 and broken line, TT, n=116). Kaplan-Meier log rank test, $\chi^2=5.208$, $p=0.0225$.

Table 3 Cox proportional hazard model to test the significance of clinical covariates and genotypes of the *CYP11B2* polymorphism as predictors of renal survival

Variable	p value	HR	95% CI
Urinary protein excretion >1.0 g/day	<0.0001	3.362	1.891 to 5.977
Hypertension	0.0007	2.301	1.423 to 3.721
No ACEI/ARB administration	0.0002	2.779	1.629 to 4.740
CC genotype of <i>CYP11B2</i>	0.1667	1.584	0.825 to 3.042

HR, hazard ratio; CI, confidence interval; ACEI/ARB, angiotensin-I converting enzyme inhibitor and/or angiotensin receptor blocker.

The Hardy-Weinberg equilibrium was tested by a chi-square test with 1 df. The time from renal biopsy to end point (initiation of dialysis or a doubling of the sCr level after the time of diagnosis) was analysed by the Kaplan-Meier method and the Cox proportional hazards regression model. Covariates were selected by a stepwise backward method and their effects were expressed as a hazard ratio. A value of $p<0.05$ was considered statistically significant.

RESULTS

In total, 271 patients with IgAN were genotyped for the *CYP11B2* C-344T polymorphism. The frequencies of the genotypes TT, TC, and CC were as follows: males, 40.6%, 50.0%, and

9.4%, respectively, females 44.8%, 44.1%, and 11.2%, respectively, and overall, 42.8%, 46.9%, and 10.3%, respectively. These results are consistent with a previous report on a large population based sample of Japanese.²⁴ The observed genotype frequencies were in agreement with those expected under the assumption of Hardy-Weinberg equilibrium. Table 1 shows the clinical characteristics of the patients and their comparisons by each genotype in women (n=143) and men (n=128). There were no statistically significant differences among the three genotypes with regard to any clinical characteristics. Blood pressures both at the time of diagnosis and during observation, as well as the incidence of hypertension at the baseline, tended to be numerically higher in patients with the CC genotype both in men and women, but the differences were not statistically significant. Duration of observation was significantly shorter in female patients with the CC genotype than those with other genotypes, but not in males.

Table 2 shows the allele frequencies of *CYP11B2* C-344T in hypertensive and normotensive subjects. In both female and male patients, there was no difference in the allele frequencies between hypertensives and normotensives.

Of 271 patients, 84 (31.0%) progressed to the primary end point (PRD). The incidence of PRD was also numerically, but not significantly, higher in patients with the CC genotype. Figure 1 shows the length of time of renal survival in each genotype. The renal survival rate was significantly worse in the CC genotype than that in other genotypes (Kaplan-Meier, log rank test, $\chi^2=5.208$, $p=0.0225$).

The Cox proportional hazard regression model was used to test further the significance of variates for progressive renal dysfunction. Although in univariate analysis the CC genotype of *CYP11B2* was a significant risk factor for PRD with a hazard ratio (HR) of 2.099 ($p=0.0249$, 95% confidence interval (CI) 1.098 to 4.013), after adjusting for other clinical risk factors, it was not recognised as an independent risk factor in the multivariate analysis. In this multivariate analysis, urinary protein of more than 1.0 g/day (HR=3.362, $p<0.0001$, 95% CI 1.891 to 5.977), hypertension (HR=2.301, $p=0.0007$, 95% CI 1.423 to 3.721), and no ACEI/ARB therapy (HR=2.779, $p=0.0002$, 95% CI 1.629 to 4.740) were identified as significant and independent risk factors for PRD (table 3). These covariates were selected by stepwise backward analysis. No other clinical variables, such as gender, age, glucocorticoids, antihypertensives other than ACEI/ARB, were selected as a significant prognostic factor by this analysis.

We next investigated the significance of these risk factors and the *CYP11B2* genotype within groups for each gender (table 4). In both female and male patients, urinary protein, hypertension, and no ACEI/ARB administration were significant risk factors. In female patients, the CC genotype of the *CYP11B2* C-344T polymorphism was also recognised as an independent risk factor (HR=4.284, $p=0.0022$, 95% CI 1.686

Table 4 Cox proportional hazard model to test the significance of clinical covariates and genotypes of the *CYP11B2* polymorphism as predictors of renal survival within groups for each gender

Variable	p value	HR	95% CI
Female patients (n=143)			
Urinary protein excretion >1.0 g/day	0.0141	2.505	1.203 to 5.217
Hypertension	0.0073	1.918	1.259 to 3.942
No ACEI/ARB administration	0.0074	3.225	1.368 to 7.599
CC genotype of <i>CYP11B2</i>	0.0022	4.284	1.686 to 10.881
Male patients (n=128)			
Urinary protein excretion >1.0 g/day	0.0036	4.768	1.664 to 13.666
Hypertension	0.0007	3.649	1.725 to 7.718
No ACEI/ARB administration	0.0214	2.299	1.131 to 4.672
CC genotype of <i>CYP11B2</i>	0.6269	0.788	0.301 to 2.061

HR, hazard ratio; CI, confidence interval; ACEI/ARB, angiotensin-I converting enzyme inhibitor and/or angiotensin receptor blocker.

Figure 2 The renal survival rate in women (A) and men (B) with each genotype of the *CYP11B2* C-344T polymorphism. (A) Renal survival in female patients with the CC genotype (solid line, $n=16$) was significantly poorer than in those with other genotypes (dotted line, $n=127$). Log rank test, $\chi^2=13.748$, $p=0.0002$. (B) In contrast, no effect of the *CYP11B2* genotype on the renal survival rate was observed in males (solid line, CC, $n=12$ and dotted line CT/TT genotype, $n=116$). Log rank test, $\chi^2=0.022$, $p=0.8832$.

to 10.881). In contrast, its hazard ratio was much lower and was not significant in male patients ($HR=0.788$, $p=0.6269$, 95% CI 0.301 to 2.061). These gender specific effects of the *CYP11B2* genotype were further confirmed by the Kaplan-Meier analysis (fig 2). Although the *CYP11B2* genotype had no effect on the renal survival rate in males (fig 2B, log rank test, $\chi^2=0.022$, $p=0.8832$), the renal survival in female patients with the CC genotype was significantly poorer than in those with other genotypes (fig 2A, log rank test, $\chi^2=13.748$, $p=0.0002$).

DISCUSSION

This study clearly shows an association of genetic polymorphism in the promoter region of *CYP11B2* with the progression of renal dysfunction. Unexpectedly, the impact of the *CYP11B2* C-344T polymorphism was strikingly observed only in female patients, with no effect at all in males. The effect of this gene polymorphism was independent of other clinical risk factors including urinary protein excretion, hypertension, and no ACEI/ARB treatment.

We could not completely exclude the possibility that the *CYP11B2* polymorphism affected renal survival partly via an effect on the blood pressure, because, although the differences were not statistically significant, patients with the CC genotype of the *CYP11B2* polymorphism consistently tended to have higher blood pressures and a higher incidence of hypertension. However, this tendency for a higher blood pressure in the CC genotype was also observed in men, in whom no effect of the genetic polymorphism was detected. Moreover, the allele frequencies were no different between hypertensive and normotensive subjects at the time of diagnosis. These observations suggest that the *CYP11B2* C-344T polymorphism affected renal prognosis via a mechanism distinct from the regulation of blood pressure. The existence of mineralocorticoid receptors has been proven in non-epithelial tissue including vascular smooth muscle cells and endothelial cells both in experimental animals and in human.^{25, 26} In addition to its actions at the collecting tubule, aldosterone can also participate in pathophysiology via effects on the heart, vasculature, and kidney. Its most significant contributions to cardiovascular disease are the result of actions at these sites rather than those related to Na and water retention.^{14, 15, 27} The role of aldosterone in the progression of renal injury has been shown,^{28, 29} and mineralocorticoid receptors are thought to mediate these actions including fibrogenesis/sclerosis in the glomerular mesangium.^{30, 31}

The mechanism by which the *CYP11B2* polymorphism affects renal survival only in female patients with IgAN is unknown. However, both in humans and animal models, it

has been well documented that males have a higher blood pressure and prevalence of end stage renal disease than age matched females.³² In the present study, a higher proportion of male patients were hypertensive as compared with female patients at the baseline, although gender was not identified as an independent risk factor for PRD and the difference was not statistically significant. The reason for this gender difference in humans is unknown, but animal models have provided insights into potential mechanisms. The sex hormones such as testosterone and androgens have been shown to have a direct effect on the renin-angiotensin-aldosterone system and on sodium reabsorption in the proximal tubule of the nephron.^{33, 34} Therefore, there is a possibility that the effect of male sex hormones reduces the sensitivity to the impact of the genetic polymorphism in *CYP11B2* in males. Conversely, the other possible explanation may be that female gender hormones such as oestrogen may be responsible for the increased sensitivity to the *CYP11B2* polymorphism in women. In fact, the increase in aldosterone level in response to angiotensin II infusion is greater in women than men.³⁵ Moreover, it has recently been reported that fluctuations in the ovarian hormones along the menstrual cycle are associated with significant variations in the homeostatic mechanisms regulating the cardiovascular system including aldosterone.³⁶ Unfortunately, the number of subjects in this study was insufficient to analyse female patients separately according to whether they were pre- or postmenopausal. Moreover, a substantial proportion of the female patients presumably experienced their climacteric phase during the observation, whereas the majority of them were estimated to be premenopausal, at least at the time of diagnosis, because the mean age of the patients was 37.0 (SD 13.4) years.

Recently, the aldosterone receptor antagonist, spironolactone, was reported to have a protective effect on cardiovascular diseases including glomerulonephritis.²⁸ The Randomized Aldactone Evaluation Study (RALES), in which a low dose of spironolactone was used as an adjuvant to conventional therapy with an ACEI, loop diuretic with or without digitalis, showed a 30% reduction in the overall risk of mortality that could not be accounted for by a blood pressure reduction or fluid loss.³⁷ The result of this study may suggest that the therapeutic efficacy of anti-aldosterone agents can be enhanced by more selective and active usage in female patients with the particular genotype of *CYP11B2* C-344T.

Although genetic polymorphism of *CYP11B2* C-344T has been reported to be associated with the serum level of aldosterone, as well as the left ventricular size, its function, and myocardial infarction independently of blood pressure,²¹⁻²³ the limitation of this study is that the association between the level of aldosterone and disease progression could not be

tested, because we have no data on the level of circulating or local tissue activity of aldosterone in each genotype group.

It also remains to be seen if the effect of the genetic variant investigated in the present study is observed in other ethnic groups. The allele frequency of *CYP11B2* C-344 in a white population was reported as 0.47 to 0.52.²² This is higher than that in the Japanese population, which was 0.34 in this study. As it is well known that a polymorphism with a higher allele frequency has more statistical power in an association study,³⁸ investigations in other ethnic groups may provide further evidence for the role of this genetic polymorphism in the progression of renal dysfunction.

In conclusion, the present study provides the first evidence for a gender specific association between the *CYP11B2* C-344T polymorphism and the prognosis of renal function in Japanese patients with IgAN. Although the genotype has no influence on renal survival in men, the CC genotype of *CYP11B2* is a possible predictive genetic marker for progression of renal dysfunction in women.

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Authors' affiliations

J Song, I Narita, S Goto, N Saito, K Omori, F Sato, J Ajiro, D Saga, D Kondo, M Sakatsume, F Gejyo, Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Niigata, 951-8510, Japan

Correspondence to: Dr I Narita, Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences, 757 Asahimachi-dori, Niigata, 951-8510, Japan; naritai@med.niigata-u.ac.jp

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